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Research Note—

Differentiation of *Mycoplasma gallisepticum* Vaccine Strains ts-11 and 6/85 from Commonly Used *Mycoplasma gallisepticum* Challenge Strains by PCR

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SUMMARY. *Mycoplasma gallisepticum* (MG) is an important avian pathogen causing significant economic losses within the poultry industry. In an effort to develop tools to aid in MG research and diagnostics, we have compared sequences of the attenuated MG vaccine strain ts-11 to those of commonly used pathogenic challenge strains in search of a simple means of differentiation. Via *gapA* sequence alignments and comparisons, we have identified and designed primers facilitating strain differentiation. When applied to conventional polymerase chain reaction (PCR) assay at low annealing temperature, the primer sets allow for the differentiation of MG attenuated vaccine strains ts-11 as well as the attenuated MG vaccine strain 6/85 from the commonly utilized MG challenge strains R_{low}, R, and S6. Conventional PCR differentiation is based on the visualization of sole products with the attenuated MG strains ts-11 and 6/85 and the lack of the corresponding products from MG strains R_{low}, R, and S6. When applied to MG strain F, product visualization varies with the applied primer set. The differentiation of MG strains ts-11 and 6/85 from the pathogenic challenge strains was also accomplished via real-time analyses, however, the primer sets were not able to differentiate MG strains ts-11 and 6/85 from selected MG field isolates.

RESUMEN. *Nota de Investigación*—Diferenciación mediante reacción en cadena por la polimerasa de las cepas vacunales de *Mycoplasma gallisepticum* ts-11 y 6/85 de las cepas de *Mycoplasma gallisepticum* comúnmente utilizadas para desafío.

El *Mycoplasma gallisepticum* es un patógeno aviar importante que causa pérdidas económicas significativas en la industria avícola. En un esfuerzo para desarrollar herramientas que ayuden en la investigación y diagnóstico del *Mycoplasma gallisepticum*, se compararon las secuencias de la cepa atenuada de *Mycoplasma gallisepticum* ts-11 con las de cepas patogénicas comúnmente utilizadas para desafío buscando formas simples para diferenciarlas. Mediante alineamientos de secuencias y comparaciones del gen *gapA*, se identificaron y diseñaron iniciadores que facilitan la diferenciación de las cepas. Cuando se utilizan en una prueba convencional de reacción en cadena por la polimerasa, los iniciadores permiten la diferenciación de las cepas vacunales atenuadas de *Mycoplasma gallisepticum* ts-11 y 6/85, de las cepas de *Mycoplasma gallisepticum* R_{low}, R, y S6, comúnmente utilizadas para desafíos. La diferenciación mediante la prueba convencional de reacción en cadena por la polimerasa se basa en la detección de un producto individual con las cepas atenuadas ts-11 y 6/85 y la carencia de los productos correspondientes con las cepas de *Mycoplasma gallisepticum* R_{low}, R, y S6. Cuando los iniciadores se utilizan con la cepa F de *Mycoplasma gallisepticum* la visualización es variable. La diferenciación de las cepas de *Mycoplasma gallisepticum* ts-11 y 6/85 de las cepas de desafío, también se realizó mediante análisis en tiempo real, sin embargo, los iniciadores no fueron capaces de diferenciar las cepas *Mycoplasma gallisepticum* ts-11 y 6/85 de cepas de campo seleccionadas de *Mycoplasma gallisepticum*.

Key words: *Mycoplasma gallisepticum*, PCR, vaccine strain, vaccine differentiation

Abbreviations: C_T = threshold cycle; FMS = Frey's media with swine serum; MG = *Mycoplasma gallisepticum*; NTC = no template control; OD = optical density; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; SDS = sequence detection software; T_m = melting temperature

Mycoplasma gallisepticum (MG) is an important avian pathogen causing economic losses to the poultry industry and most significantly impacting the egg layer industry. MG is the etiologic agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys. Though the pathogenesis concerning avian species is not well understood, the consequences of MG infections can be significant and include increased rates of mortality and carcass condemnation and reduced egg production, hatchability, feed efficiency, and weight gain (4,24,28). To protect against outbreaks by MG and other pathogens, intense biosecurity practices have been implemented throughout the poultry industry. In addition, rigid biosurveillance via serological monitoring, MG isolation, and DNA-based detection is routine (18,19,28). Within the layer egg industry, three live attenuated MG vaccine strains have been approved: F (FVAX-MG®; Schering-Plough Animal Health, Omaha, NE), 6/85 (MYCOVAC-L®; Intervet Inc., Millsboro, DE), and ts-11 (MG vaccine®; Select Laboratories, Gainesville, GA). Further, these vaccine strains have been shown to reduce the impact of MG infection (1,4,8,13,32).

The continued occurrence of MG outbreaks in spite of current control strategies and the endemic nature of MG as associated with large multiage layer facilities necessitates the development of a novel effective means of MG control (7). However, this development is currently hampered by the lack of knowledge associated with MG and MG-induced pathogenesis and the availability of applicable tools. Even the means by which currently available vaccine strains impart protection to their host remains a subject of debate and an understanding of this process could aid in the development of novel control strategies. Historically, MG vaccine-related research focused

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                 -----PRUMG32-F-------->
                        -----PRUMG30-F------->
ts-11     TTAACTAGAACTGATTTTGGAAGCGTAAGATCTGAAACTCAAGATGCAAATACCGTATCATCTAAATTAAACGGCGCTTACTTATCATCA   2324
S6        TTAACTAGAACTGATTTTGGAAGTACTACTCCTGCAACTCAAGATGCAAATACCGTATCATCTAAATTAAACGGCGCTTACTTATCATCA   1815
Rlow      TTAACTAGAACTGATTTTGGAAGTACTACTCCTGCAACTCAAGATGCAAATACCGTATCATCTAAATTAAACGGCGCTTACTTATCATCA   2305

            <-----PRUMG36-R------------
ts-11     ACAGGAGATTCA---GGATGATATAACGGTTCAATCTATGTTAAACAAGCAAACTTTACACCAAGTAGCCAAGGTTATACTTGACAAGAT   2411
S6        ACTGGCGATCAACAAGGATGATACAACGGTTCAATCTATGTTAAAAAAGCGAGCTTTACACCAAGTAGTCAAGGTTATACTTGACAAGAT   1905
Rlow      ACTGGCGATCAACAAGGATGATACAACGGTTCAATCTATGTTAAAAAAGCGAGCTTTACACCAAGTAGCCAAGGTTATACTTGACAAGAT   2395

                                                 <-----PRUMG31-R------
ts-11     TTCAAAGGTTTAACAACTACAGCAAGTAACGCAGTTATTTCTAACTGAACAAAAGCCGGATACAGTATTAGACCAGATGATGATACAGTA   2501
S6        TTCAAAGGTTTAACAACTACAGCAAGTAACGCAGTTATTTCTAACTGAACAAAAGCTGGATACAGTATTAGACCAGATGATGATACAGTA   1995
Rlow      TTCAAAGGTTTAACAACTACAGCAAGTAACGCAGTTATTTCTAACTGAACAAAAGCTGGATACAGTATTAGACCAGATGATGATACAGTA   2485
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Fig. 1. CLUSTAL sequence alignments of partial sequences of the *gapA* gene of *M. gallisepticum* strains S6 (AF083976), R_{low} (AE015450), and ts-11 (AY212515). Primer orientation is presented and highlighted nucleotides indicate sequence differences within regions selected for primer design.

on vaccine efficacy following a virulent MG challenge and protection was characterized in terms of egg production, disease pathology, or host immune response. The resulting MG populations (vaccine strain *vs.* challenge strain) were not addressed due to the lack of available tools and the complexities of MG strain differentiation. As this information is necessary to describe the protective mechanism of currently available live MG vaccines, this report describes MG vaccine strain-specific primer sets applicable to simple one-step conventional and the more time-efficient real-time polymerase chain reaction (PCR) systems which allow the identification of the MG vaccine strains ts-11 and 6/85 alone or in the presence of the commonly utilized laboratory challenge MG strains R, R_{low}, and S6.

MATERIAL AND METHODS

Mycoplasma gallisepticum strains. MG strains R and S6 were obtained from laboratory stocks. MG strain R_{low} (13 passages) was kindly provided by Dr. Steven Geary (University of Connecticut). MG vaccine strain 6/85 was obtained from Intervet Inc., MG vaccine strain ts-11 from Merial Select (Gainesville, GA), and MG strain F from Schering-Plough Animal Health. Field isolate strains K4043, K4421A, K4465, K5089, K5111A, and K5234 were kindly provided by Dr. Stanley Kleven and have been previously described (11,13,20,31).

Culture and sample preparation. All MG strains were propagated in Frey's media with swine serum (FMS) broth media (29) supplemented with 35 ml of Yeast Extract Solution per liter (#18180-059, Invitrogen/Gibco, Grand Island, NY) at 37 C. Upon color indicator (phenol red) change (>18 hr), 1 ml aliquots were stored at −80 C. For DNA isolation, genomic DNA was extracted using the InstaGene Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Briefly, cell pellets resulting from 1 ml of culture were washed in 150 mM (pH 7.2) phosphate-buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) and resuspended in 200 µl InstaGene matrix. Following a 30 min incubation at 56 C, cell suspensions were boiled (100 C, 10 min), and centrifuged (20,000 × *g*, 5 min, 4 C). The resulting supernatants were stored (−80 C) for subsequent use.

Primer design. The *gapA* sequences from commonly used MG challenge strains S6 and R_{low} and MG vaccine strain ts-11 were obtained from GenBank. Alignments were performed using CLUSTALW, version 1.83 (Fig. 1) (30). Dissimilar regions were manually identified and employed to design primers specific for MG vaccine strain ts-11 utilizing Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Oligonucleotide cross-reactivity was assessed by BLASTn analyses (2). Primer sequences are as follows:

PRUMG30-F: 5′-TTTGGAAGCGTAAGATCTGAAA-3′ (nt: 2250–2271)
PRUMG31-R: 5′-TCCGGCTTTTGTTCAGTTAGA-3′ (nt: 2451–2471)
PRUMG32-F: 5′-GAACTGATTTTGGAAGCGTAAGA-3′ (nt: 2242–2264)
PRUMG36-R: 5′-CCGTTATATCATCCTGAATCTCCT-3′ (nt: 2327–2350)

Nucleotide positions of the primers are numbered according to the GenBank AY212515 sequence.

PCR. For conventional PCR, reactions were performed using an iCycler® (Bio-Rad Laboratories) thermocycler. Each 50 µl conventional PCR amplification contained 0.2 mM of each dNTP (Sigma Aldrich, St. Louis, MO), 1× Green GoTaq® Reaction Buffer (Promega, Madison, WI), 0.5 µM of each primer, 1.25 U of GoTaq® DNA polymerase (Promega), and 1 µl of template solution. For MG-specific conventional PCR utilizing the primers Mg14F (5′-GAG CTA ATC TGT AAA GTT GGT C-3′) and Mg13R (5′-GCT TCC TTG CGG TTA GCA AC-3′) (17), the reaction program included an initial denaturization step (95 C, 3 min) followed by a three-step cycle (35×) including a denaturization step (94 C for 30 sec), an annealing step (55 C for 30 sec), and an extension step (72 C for 1 min) and was completed with a final extension step (72 C for 7 min). For MG strain ts-11 and 6/85-dependent conventional PCR, the protocol was modified to included annealing temperatures of 55 C, 60 C, or 62.5 C. No template controls (NTCs) were included with each run.

For real-time PCR, reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems). Each 50 µl real-time PCR amplification contained 1× SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µM of each primer, and 1 µl of template solution. NTCs were included with each run. PCR programs for real-time PCR were as those used for conventional PCR except that an additional incubation (50 C for 2 min) was included prior to the initial denaturization step, the initial denaturization step was extended to 10 min to allow for polymerase activation, and the annealing temperature was maintained at 62.5 C. The PCR amplification included 40 cycles and product formation was monitored via fluorescence during the extension cycle. Following amplification, dissociation curve analyses were performed starting at 62.5 C and proceeding at a rate of 0.1 C/sec to determine melting temperature (T_m) values of the PCR products and to detect

Table 1. Conventional PCR differentiation of MG strains ts-11 and 6/85 from MG strains R, R_{low}, and S6.

Template	Annealing temperature (C)	PRUMG30-F/PRUMG31-R Bands (n)[A]	PRUMG30-F/PRUMG31-R Size (bp)[B]	PRUMG32-F/PRUMG31-R Bands (n)	PRUMG32-F/PRUMG31-R Size (bp)	PRUMG30-F/PRUMG36-R Bands (n)	PRUMG30-F/PRUMG36-R Size (bp)	PRUMG32-F/PRUMG36-R Bands (n)	PRUMG32-F/PRUMG36-R Size (bp)	Mg14F/Mg13R Bands (n)	Mg14F/Mg13R Size (bp)
ts-11	55	1	≈220	1	≈230	1	≈100	1	≈110	1	≈190
	62.5	1	≈220	1	≈230	1	≈100	1	≈110	—[C]	—
6/85	55	1	≈220	1	≈230	1	≈100	1	≈110	1	≈190
	62.5	1	≈220	1	≈230	1	≈100	1	≈110	—	—
F	55	1	≈220	1	≈230	0	—	0	—	1	≈190
	62.5	1	≈220	0	—	0	—	0	—	—	—
R	55	0	—	0	—	0	—	0	—	1	≈190
	62.5	0	—	0	—	0	—	0	—	—	—
R_{low}	55	0	—	0	—	0	—	0	—	1	≈190
	62.5	0	—	0	—	0	—	0	—	—	—
S6	55	0	—	0	—	0	—	0	—	1	≈190
	62.5	0	—	0	—	0	—	0	—	—	—
NTC	55	0	—	0	—	0	—	0	—	0	—
	62.5	0	—	0	—	0	—	0	—	—	—

[A]Bands visualized via gel electrophoresis.
[B]Band size estimated by comparison to 100 bp DNA ladder.
[C]MG-specific PCR was not conducted at the 62.5 C annealing temperature.

nonspecific amplification products. Threshold cycle (C_T) and baseline were determined automatically by the SDS version 1.3.1 software (Applied Biosystems).

Gel electrophoresis. PCR products were visualized via gel electrophoresis on a 1.5% agarose gel containing GelStar nucleic acid stain (Lonza Rockland, Inc., Rockland, MD) and visualized on a ChemiDoc system (Bio-Rad Laboratories). The size of the amplified product(s) was estimated via comparison to a 100 bp DNA molecular weight ladder (Promega).

DNA quantification. Concentrations of DNA from MG strains were calculated by measuring optical density (OD) at 260 and 280 nm with a BioRad SmartSpec™ 3000 (Bio-Rad Laboratories). Briefly, DNA was measured at two dilutions and the average of resulting concentrations was utilized.

Clinical samples. Hyline Commercial W-36 laying hens ($n = 3$) originating from a certified MG- and *Mycoplasma synoviae*-free commercial source at 1 day of age were used for clinical evaluation of the PRUMG32-F/PRUMG36-R primer set. Through 60 wk of age, the birds were maintained in an environmentally controlled facility. At 55 wk of age, the hens were transferred to an environmentally controlled disease isolation facility and placed within a negative pressure isolator unit. The birds were tested for exposure to MG via serum plate agglutination as previously described (6). The hens were vaccinated with MG strain 6/85 at 56, 65, and 72 wk of age. At 77 wk of age, tracheal swabs were collected from each bird, the swabs were pooled in 1 ml PBS, genomic DNA was isolated from the resulting suspension via the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and the resulting supernatants were stored (−80 C) for subsequent use. Real-time PCR was carried out with a 60 C annealing temperature with the PRUMG32-F/PRUMG36-R and MG-specific primer sets (17).

RESULTS

Due to the availability of strain-specific primers for MG strain F (25) and a real-time primer/probe assay for the differentiation MG strain 6/85 from a MG challenge strain (10), only the *gapA* sequence of MG vaccine strain ts-11 (AY212515) and those of MG challenge strains S6 (AF083976) and R_{low} (AE015450) were originally considered for the design of the primers. Sequence alignments revealed a region of variation (66% heterogeneity) within a short (12 bp) region of the *gapA* sequence of MG strain ts-11 (nt 2258–nt 2269) as compared to sequences from MG strains S6 and R_{low} (Fig. 1). Utilizing Primer Express 3.0 software, two primers (PRUMG30-F and PRUMG32-F) were designed to encompass this region. Due to a lack of additional regions of sequence variation, reverse primers were designed within a region of significant interstrain homology (PRUMG31-R) and within a region containing a 3 nt gap within MG strain ts-11 sequence as compared to that of MG strains R_{low} and S6 (PRUMG36-R). All primers were designed with respect to conservation of the DNA sequence within the MG strain ts-11 *gapA* gene and were predicted to amplify products of 222, 230, 101, and 109 bp in size for the primer pairings of PRUMG30-F/PRUMF31-R, PRUMG32-F/PRUMF31-R, PRUMG30-F/PRUMF36-R, and PRUMG32-F/PRUMF36-R, respectively. Sequence comparisons and primer locations are shown in Fig. 1.

Prior to testing primer sets, template solutions were quantified via spectrophotometer and the template quality was verified with all templates using the MG-specific PCR assay of Lauerman (17). MG-specific conventional PCR assays resulted in the visualization of a ≈190 bp product for all templates (21) (Table 1). Initial conventional PCR assays to test primers sets against MG templates were designed for low stringency with a 55 C annealing temperature. Results from reactions utilizing primer sets PRUMG30-F/PRUMG31-R and PRUMG32-F/PRUMG31-R demonstrated product formation at this low annealing temperature for MG strain ts-11, 6/85, and F templates, but not for templates of MG strains R, R_{low}, or S6 (Table 1). The gel resulting from the PRUMG32-F/PRUMG31-R primer set is shown in Fig. 2. Reactions (annealing temperature = 55 C) with the primer sets PRUMG30-F/PRUMG36-R and PRUMG32-F/PRUMG36-R similarly yielded no visible product with templates from MG strains R, R_{low}, and S6 (Table 1). Further, while these primer sets yielded appropriately sized products for MG strains ts-11 and 6/85 (as predicted from MG strain ts-11 sequence), there was no product visualized with MG strain F template with these primer sets (Table 1). To raise the stringency of the conventional PCR assays, the annealing temperatures were increased. At the 60 C annealing temperature, no difference in product visualization was demonstrated for any of the primer sets as compared to the results at

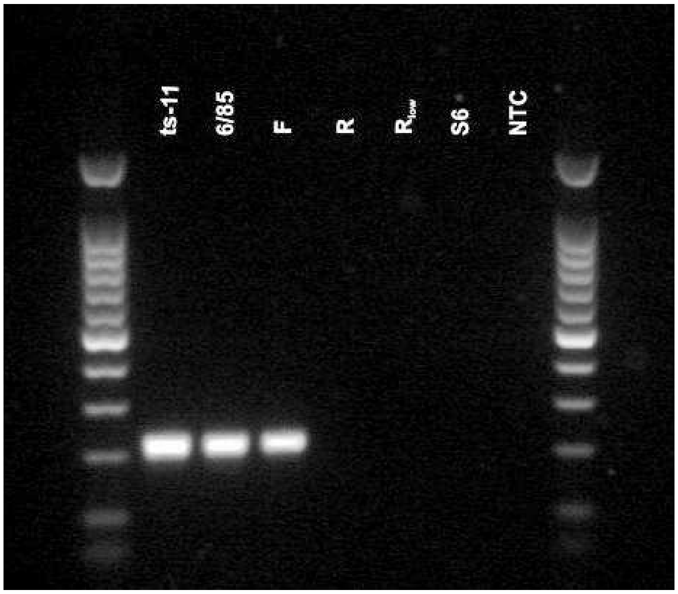

Fig. 2. MG vaccine strain-specific conventional PCR amplification utilizing the primer set PRUMG32-F/PRUMG31-R. Lane 1, 100 bp DNA ladder; Lane 2, MG strain ts-11; Lane 3, MG strain 6/85; Lane 4, MG strain F; Lane 5, MG strain R; Lane 6, MG strain R_{low}; Lane 7, MG strain S6; Lane 8, no template control (NTC); Lane 9, 100 bp DNA Ladder.

the lower annealing temperature (data not shown). However, the annealing temperature of 62.5 C did result in product alterations. While products were visualized for the MG strains ts-11 and 6/85 template with all primer sets, only the PRUMG30-F/PRUMG31-R yielded visible product for MG strain F at the 62.5 C annealing temperature (Table 1).

When applied to the 7500 Real-Time PCR System, application of the primer sets demonstrated results similar to those of conventional PCR. As per conventional PCR assays, verification of template quality was accomplished by applying MG-specific primers Mg14F and Mg13R (17) to the real-time PCR system (10,23). Results demonstrated amplification of all templates with MG-specific primers and dissociation curve analyses indicated production of a single product ($T_m \approx 81.0$ C) (Table 2). When primer sets were applied to the real-time system, product was consistently detected with templates of MG strains ts-11 and 6/85, but no products were observed for the pathogenic challenge strains R, R_{low}, or S6 (Table 2). In regards to MG strain F template, amplified products were detected for the PRUMG30-F/PRUMG31-R and PRUMG32-F/PRUMG31-R primer sets, but no products were detected for this template with the primer sets PRUMG30-F/PRUMG36-R or PRUMG32-F/PRUMG36-R (Table 2). The amplification plot for the primer set PRUMG32-F/PRUMG36-R is shown (Fig. 3).

Due to the apparent increased specificity (as indicated by failure of product formation with MG strain F associated with the primer PRUMG36-R and the heterogeneity located at the 3′ end of the primer PRUMG32-F), the primer set PRUMG32-F/PRUMG36-R was selected for further analyses. To compare the sensitivities of the PRUMG32-F/PRUMG36-R primer set to MG strain ts-11 and 6/85 templates, template DNA concentrations from these MG strains were normalized to 40 µg/ml. A series of 10-fold dilutions was made and applied to conventional (annealing temperature = 60 C or 62.5 C) and real-time (annealing temperature = 62.5 C) PCR. At the 60 C annealing temperature, conventional PCR results indicated product formation for MG strain ts-11 and 6/85 templates at the 10^{-3} dilution (40 pg DNA per reaction) and 10^{-4} dilution (4 pg DNA per reaction), respectively (data not shown). At the 62.5 C annealing temperature, conventional PCR products were visible at the 10^{-3} dilution (40 pg DNA per reaction) for both MG strain ts-11 and 6/85 templates, however band intensity appeared to be lower than that associated with the 60 C annealing temperature (data not shown). When the diluted DNA templates were applied to real-time PCR (annealing temperature = 62.5 C), products were detected at the 10^{-4} dilution (4 pg DNA per reaction) via SDS software for MG strain ts-11 and 6/85 templates. The associated amplification plots for MG strains ts-11 and 6/85 are offered as Fig. 4A and B, respectively. To test the applicability of this primer set concerning detection of MG strains 6/85 and ts-11 in mixed-strain samples, real-time reactions for MG strain 6/85 and ts-11 templates (40 ng/reaction) were spiked with R strain template (85 ng/reaction). While the presence of R strain template was associated with slight increase ($\approx$2 cycles) in C_T, no difference in product melting temperature ($T_m = 73.8$ C) was observed (data not shown).

To assess the applicability of the PRUMG32-F/PRUMG36-R primer set on clinical samples, template derived from tracheal swabs of MG strain 6/85–vaccinated birds were applied to real-time assays with the PRUMG32-F/PRUMG36-R primer set and the MG-specific primer set of Lauerman (17). Amplified product was detected with both primer sets, computed C_T values were similar (35.16 *vs.* 37.22

Table 2. Real-time differentiation of MG strains ts-11 and 6/85 from MG strains R, R_{low}, and S6.

Primer set		Template						
		ts-11	6/85	F	R	R_{low}	S6	NTC
PRUMG30-F/	$C_T^{A,B}$	16.6 ± 0.09	15.7 ± 0.23	28.59 ± 0.09	undet.[C]	undet.	undet.	undet.
PRUMG31-R	$T_M^{A,B}$	76.9 ± 0	77.05 ± 0.15	76.7 ± 0.2	—[D]	—	—	—
PRUMG32-F/	$C_T^{A,B}$	15.85 ± 0.1	15.08 ± 0.37	22.725 ± 0.305	undet.	undet.	undet.	undet.
PRUMG31-R	$T_M^{A,B}$	76.9 ± 0	76.9 ± 0	76.5 ± 0	—	—	—	—
PRUMG30-F/	$C_T^{A,B}$	16.97 ± 0.04	14.75 ± 0.07	undet.	undet.	undet.	undet.	undet.
PRUMG36-R	$T_M^{A,B}$	74.7 ± 0	75.1 ± 0	—	—	—	—	—
PRUMG32-F/	$C_T^{A,B}$	16.12 ± 0	13.665 ± 0.16	undet.	undet.	undet.	undet.	undet.
PRUMG36-R	$T_M^{A,B}$	74.7 ± 0	74.9 ± 0.2	—	—	—	—	—
Mg14F/Mg13R	$C_T^{B,E}$	16.86 ± 0.23	14.49 ± 0.16	15.76 ± 0.2	16.67 ± 0.47	15.69 ± 0.4	16.04 ± 0.05	undet.
	$T_M^{B,E}$	81.0 ± 0	81.0 ± 0	81.0 ± 0	81.0 ± 0	81.0 ± 0	80.8 ± 0.1	—

[A]Mean of duplicate reactions.
[B]Plus or minus standard error of the mean.
[C]Undet. = undetected.
[D]Dashes (—) indicate unreported value due to undeterminable product.
[E]Mean of triplicate reactions.

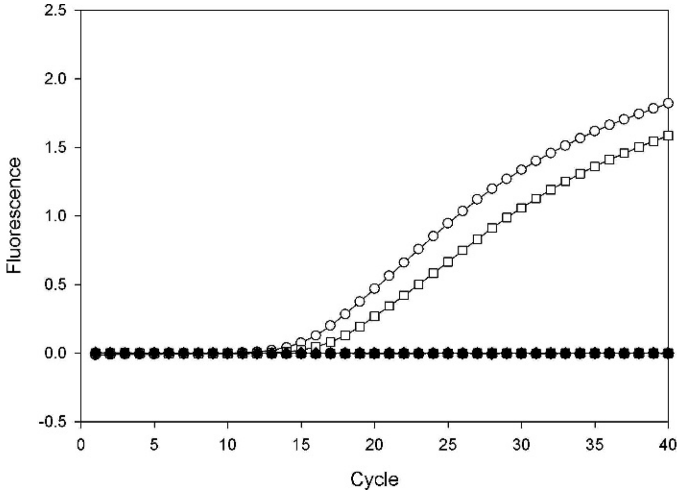

Fig. 3. Real-time PCR fluorescent-amplification profile depicting specificity of MG strain-specific PCR assay utilizing the primer set PRUMG32-F/PRUMG36-R and templates from MG strains ts-11 (□), 6/85 (○), F (■), R (▲), R_{low} (△), and S6 (◇). Reaction also includes a no template control (NTC) (●). Fluorescent emissions are plotted as a function of cycle number.

for MG-specific PCR and PRUMG32-F/PRUMG36-R PCR, respectively), and dissociation curve analyses yielded T_m values of 82.0 C and 75.1 C respectively. The applicability of the PRUMG32-F/PRUMG36-R primer set for differentiation of MG strains ts-11 and 6/85 from field strain isolates was also assessed. Field isolates selected for study included MG isolates K4043, K4421A, K4465, K5089, K5111A, and K5234 that were previously determined to be genetically similar to MG strain 6/85 (11,13,20,30). The reactions were performed via real-time PCR (annealing temperature = 62.5 C) and results indicated 100% product detection for the field isolates. Further analyses indicated that all field isolates shared a product dissociation temperature of 73.8 C (data not shown).

DISCUSSION

The efficient and accurate differentiation of MG strains is important in vaccine trials to evaluate various vaccination programs (13), to determine MG strain interactions, and describe the protection afforded by currently available live MG vaccines, an area of importance limiting the development of further means of control. The ability to differentiate MG strains is also fundamental to controlling spread of the avian pathogen within the poultry industry. Within the egg layer industry alone, the ability to discern between the widely used attenuated vaccine strains of MG and virulent field strains is critical in recognizing field challenges (18). Various techniques to differentiate MG strains have been previously described (11,13,26) and include fingerprinting techniques such as restriction fragment length polymorphism (3,15,16,21,27), ribotyping (33), pulsed-field gel electrophoresis (22), amplified length polymorphism (13), and random amplified polymorphic DNA (5,9,12,13). However, applicability of these techniques may be limited by a requirement for pure cultures. More recently, chromosomal loci sequencing has been applied to MG strain differentiation and while strain discrimination was demonstrated (11,26), the technical, monetary, and time requirements may limit the widespread application of this technique particularly within research projects involving a large number of samples.

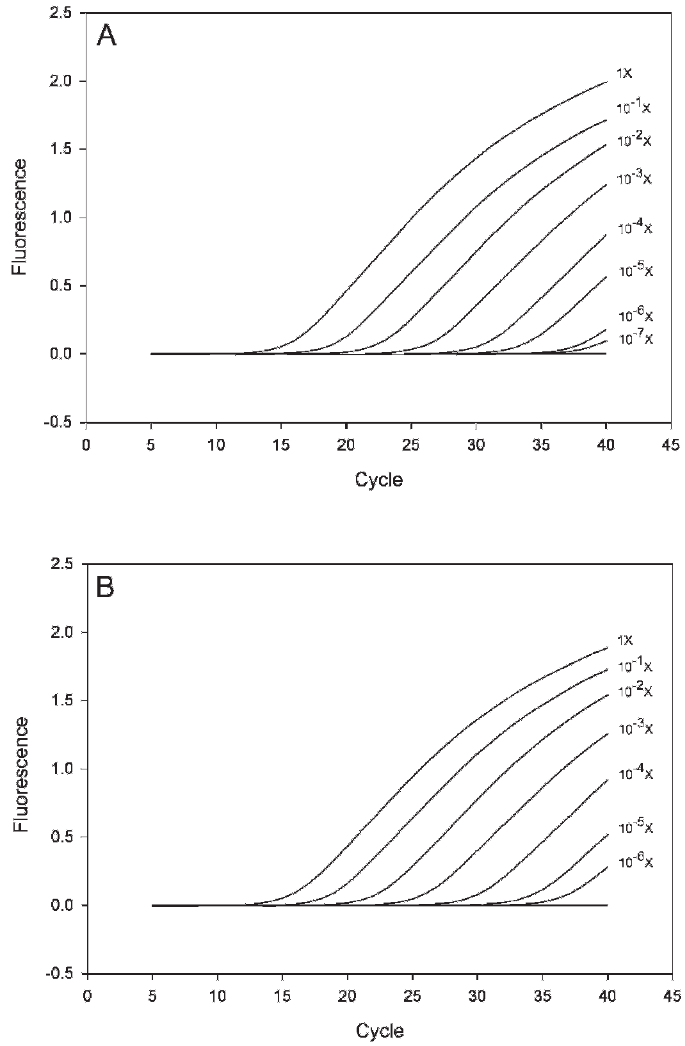

Fig. 4. Real-time PCR fluorescent-amplification profile depicting sensitivity of MG strain-specific PCR assay utilizing the primer set PRUMG32-F/PRUMG36-R and templates from MG strains (A) ts-11 and (B) 6/85. Ten-fold serial dilutions of DNA (1× = 40 μg/ml) were utilized. Fluorescent emissions are plotted as a function of cycle number. Amplification plots correspond to 1× (left-most), 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} template dilutions.

Previously, the discriminatory power of MG strain-specific primers and probes has been demonstrated (10,14,25). Advantages of this system for PCR-based vaccine strain identification include timely diagnostics, technical ease, and applicability to both pure and complex (mixed) samples. However to date, no such strain-specific primer set or probe has been developed for MG vaccine strain ts-11. Results reported herein indicate that all of the described primer sets may be applied to differentiate the live attenuated MG vaccine strain ts-11 as well as MG vaccine strain 6/85 from the MG laboratory challenge strains R, R_{low}, and S6 via one-step conventional and real-time PCR assays. Regarding template from MG strain F, product detection/visualization varied according to primer set applied. With conventional PCR (annealing temperature = 55 C or 60 C) and real-time assays (annealing temperature = 62.5 C), product from MG strain F template was detected/visualized with the primer sets including either of the forward primers PRUMG30-F and PRUMG32-F and the reverse primer PRUMG31-R, but not with reactions including the PRUMG36-R primer. The findings can be rationalized, however, as sequence analyses revealed marked similarity between primers PRUMG30-F, PRUMG31-R, and PRUMG32-F primers and the partial *gapA* sequence of MG F strain (AY227006). In contrast, sequence comparison of the PRUMG36-R primer to F strain sequence revealed the 3 nt gap previously associated with *gapA* sequences from MG strains R, R_{low}, and S6 possibly underlying the exclusion of product formation in reactions involving this primer PRUMG36-R (data not shown).

The discrepancies in product detection at the 62.5 C annealing temperature with conventional and real-time assays may be attributed to the increased sensitivity as predicted from the use of the SYBR Green fluorescence detection as opposed to band visualization following gel electrophoresis. This increased level of detection was also demonstrated by the real-time PCR system when assessing the sensitivities of the PRUMG32-F/PRUMG36-R primer set to the MG strain ts-11 and 6/85 templates at the 62.5 C annealing temperature.

As reactions with field isolate templates resulted in 100% product formation, the field application of the PRUMG32-F/PRUMG36-R primer set to field diagnostics may be limited. However, it should be stressed that the field isolates chosen for assessment have all been previously shown to share a high level of sequence similarity to MG strain 6/85 (11,13,20,30) and therefore may explain apparent primer annealing and subsequent product formation from these templates. These isolates were purposefully included in the study as field applicability necessitates the ability to discern MG strains ts-11 and 6/85 from all field strains including those sharing a high degree of genetic similarity.

These findings do not however limit the applicability of this and other primer sets mentioned in this study in research-associated tasks wherein the ability to quickly and easily detect the presence of the MG vaccine strains ts-11 and 6/85 when associated with the challenge strains R, R_{low}, or S6 will provide a valuable research tool and aid in the describing the protective mechanism of currently available live MG vaccines.

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